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Antigenotoxic effects of quercetin, rutin and ursolic acid on HepG2 cells: Evaluation by the comet assay

Authors: *A.A. Ramos[1,3], C.F. Lima[1], M.L. Pereira[2], M. Fernandes-Ferreira[3], C. Pereira-Wilson[1*]*

[1]CBMA - Molecular and Environment Biology Centre/Department of Biology, University of Minho, 4710-057 Braga, Portugal

[2]Department of Biology, CICECO, University of Aveiro, 3810-193 Aveiro, Portugal

[3]CMPBP - Centre of Molecular Physiology and Biotechnology of Plants/Department of Biology, University of Minho, 4710-057 Braga, Portugal

Corresponding author: * Cristina Pereira-Wilson; telephone +351 253604318; fax +351 253678980; e-mail cpereira@bio.uminho.pt

Abstract

In the present study, the chemoprotective effects of quercetin, rutin and ursolic acid on *tert*-butyl hydroperoxide (*t*-BHP)-induced DNA damage in a human hepatoma cell line (HepG2) were investigated by the comet assay. To determine whether protection was due to direct chemical interactions alone or to cellular mediated responses three different types of treatments were used: simultaneous incubation of cells with individual test compounds and the toxicant; pre-treatment with test compound before addition of the toxicant followed or not by a recovery period. The expression of Hsp70 was quantified by Western blotting to test the involvement of heat shock proteins in the cellular responses to the test compounds. In addition, effects on proliferation were evaluated by the MTT assay. The results show that quercetin and ursolic acid prevented DNA damage and had antiproliferative properties in HepG2 cells suggesting an anticarcinogenic potential for these compounds. The protective effects of quercetin against *t*-BHP induced DNA damage seem to be due to both direct effects on *t*-BHP toxicity and to cellularly mediated indirect effects which reflect the potentiation of the the cellular antioxidant defenses. Ursolic acid seems to exert effects only through cellularly mediated mechanisms since it was not protective in simultaneous incubation. Quercetin and ursolic acid also showed to increase the rate of DNA repair. Rutin did not have effects at any level. These results, obtained with liver cells, emphasize and confirm the chemopreventive potential of quercetin and ursolic acid, which may help explain the lower cancer incidence in human population with high dietary intakes of fruits and vegetables. These results also demonstrate that Hsp70 is not involved in the observed effects in HepG2.

Keywords: Quercetin; Rutin; Ursolic Acid; HepG2 cells; Comet assay; Antigenotoxic effects

1. Introduction

Human cells are continuously exposed to reactive oxygen species (ROS) of both endogenous and exogenous sources. When excessive amounts of ROS are produced, a disturbance in the pro-oxidant/antioxidant balance in favour of the pro-oxidant state may occur, which may lead to cell damage (Valko et al., 2006). In particular, DNA damage combined with insufficient DNA repair are known to be associated with carcinogenesis (Olinski et al., 2002; Klaunig and Kamendulis, 2004). Thus, prevention of such damage or induction of repair could prevent the carcinogenic process.

Considerable epidemiological evidence suggests that diets high in fruit and vegetables are inversely related to risk of degenerative diseases such as coronary artery disease and certain cancers (Stanner et al., 2004; Collins A.R., 2005). Flavonoids, such as quercetin (Q) and rutin (R), are among the most abundant antioxidant compounds in vegetables and fruits (Aherne and O'Brien, 2002). Ursolic acid (UA) is a pentacyclic triterpenic acid that also occurs naturally in a large variety of vegetarian foods and medicinal plants (Liu, 1995). Q, R and UA are among the plant compounds to which hepatoprotective activities have been attributed (Joyeux et al., 1990; Martin-Aragon et al., 2001; Janbaz et al., 2002). However, in spite of their common hepatoprotective properties, Q and R (one of quercetin's glycosides) have high free radical scavenging activity (Ross and Kasum, 2002) whereas the more lipophilic triterpenoid UA is virtually inactive as free radical scavenger (Lo et al., 2002). This suggests different mechanisms of action for the 3 compounds. These compounds have also been reported as antigenotoxic in various *in vitro* models (Aherne and O'Brien, 2000a; Russo et al., 2000; Lo et al., 2002; Ross and Kasum, 2002).

Natural compounds play important roles in multiple mechanisms, which may be responsible for their anticarcinogenic effects. Antioxidant activity and iron quelating activities as well as inhibition of bioactivacting (phase I) enzymes and induction of detoxifying (phase II) enzymes (De Flora, 1998; Marchand, 2002; Galati and O'Brien, 2004) may provide protection against cancer initiation (antigenotoxic effects). Natural compounds may also contribute to cancer prevention by modulating DNA repair systems (De Flora, 1998; Collins et al., 2003). In addition, inhibition of proliferation of damaged cells would also be beneficial.

Recently some studies have suggested the participation of heat shock proteins, mainly Hsp70, in the induction of DNA repair (Kenny et al., 2001; Mendez et al.,

2003a; Niu et al., 2006) through effects on apurinic/apyrimidinic endonuclease (Kenny et al., 2001; Mendez et al., 2003b) and polymerase β (Mendez et al., 2003a). Hsp may also be involved in prevention of DNA damage since depletion of Hsp70 associated to oxidative stress has also been shown to induce lysosomal membrane permeabilization and release of redox-active iron, which may contribute to DNA damage (Nylandsted et al., 2004; Doulias et al., 2007).

Organic hydroperoxides, and in particular model compounds such as *tert*-butyl hydroperoxide (*t*-BHP), have been shown to cause oxidative DNA damage by promoting the formation of alkaline labile sites and single strand breaks (Guidarelli et al., 1997). Iron dependent mechanisms seem to be involved (Sestili et al., 1998). This type of damage is easily monitored by the comet assay (SCGE) single cell gel electrophoresis (Lima et al., 2006).

The aim of this study was to evaluate by the comet assay the effects of Q, R and UA (Fig. 1) on *t*-BHP-induced DNA damage, in HepG2 cells. To differentiate mechanisms potentially involved in the antigenotoxic properties of the test compounds, three types of treatment were used: 1) simultaneous treatment (cells exposed simultaneously to test compounds and *t*-BHP) where effects include direct chemical interaction between test compounds and toxicant; 2) pre-treatment (cells incubated with test compounds for 24 h before exposure to the toxicant) and 3) pre-treatment with recovery period (cells incubated as above and followed by a 2 h recovery period in fresh medium) to determine the contribution of intracellular effects induced in response to the test compounds. In view of the possible involvement of Hsp's in the induction of DNA repair enzymes and in lysosomal stabilisation, we also evaluated effects on Hsp70 expression in response to the test compounds. The effects on cell proliferation were also tested.

2. Material and methods

2.1. *Chemicals*

Quercetin, rutin, ursolic acid, *tert*-butyl hydroperoxide, HEPES (*N*- [2-hydroxyethyl] piperazine-*N'*- [2-ethane-sulfonic acid]), Minimum Essential Medium Eagle (MEM), antibiotic-antimycotic solution, trypsin solution, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Bradford Reagent, Monoclonal anti-HSP70 (clone BRM-22)

and monoclonal anti-HSP70 (clone C92F3A-5) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Stressgen Biotechnologies, Corp. (Victoria, B.C., Canada), respectively. Peroxidase-conjugated goat anti-mouse antibody, and ECL western blotting detection reagent were purchased from GE Healthcare, UK. Fetal Bovine Serum was purchased from Biochrom KG (Berlin, Germany). All others reagents were of analytical grade.

2.2. *Antiradical activity*

The free radical scavenging (antiradical) activity of the tested compounds was studied against the stable free radical DPPH as elsewhere (Lima et al., 2006). Trolox was used in this experiment as a positive control.

2.3. *Cell culture*

The HepG2 cell line was obtained from ATCC and cultured in 75 cm^2 polystyrene flasks (Falcon) with MEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate, 10 mM HEPES and 1.5 g/l sodium bicarbonate under an atmosphere of 5% CO_2 at 37ºC. For the following experiments the test compounds were dissolved in dimethylsulfoxide (DMSO - final concentration below 1%) and controls received DMSO only. *tert*-Butyl hydroperoxide was dissolved in cell culture medium.

2.4. *Cell toxicity and proliferation assays*

The test compound's cytotoxicity was assayed in 24-multiwell culture plates seeded with 1.25×10^5 cells/well. Twenty four hours after plating, test compounds were added and 24 h and 72 h later cytotoxicity was estimated by the lactate dehydrogenase (LDH) leakage assay according to the method previously described (Lima et al., 2005). Results were expressed as percentage of cell viability from the control (cells without any test compound). From this, concentrations of test compound without cytotoxicity were selected.

Effects on cell proliferation were tested in 96-multiwell culture plates at a density of 7.5×10^3 cells/well. Twenty four hours after plating, the medium was discarded and fresh medium containing test compounds at different concentrations was added. The MTT test was performed at the end of two incubation regimes: 1) a period of 24 h of incubation with compounds, followed by a period of 48 h with fresh culture

medium without tested compounds; 2) a period of 72 h with the tested compounds. The number of viable cells in each well was estimated by the cell capacity for reduction of MTT as described by Silva et al. (2006). The results were expressed as percentage of cell proliferation relative to control (cells without any test compound). For that, MTT reduction at the beginning of incubation (t = 0 h), was subtracted from all the experimental conditions used above, including the control.

2.5. E*ffects on DNA (Antigenotoxic effects)*

To study the genotoxic effects of *t*-BHP, HepG2 cells were plated for 16 h after which the medium was discarded and medium containing *t*-BHP at different concentrations (0-1000 μM) was added. One hour after, the genotoxic effects of *t*-BHP were evaluated by alkaline version of the comet assay. Incubations with 200 μM for 1 h were chosen to evaluate the protection conferred by the test compounds quercetin (Q), rutin (R) and ursolic acid (UA).

To study the antigenotoxic effects of Q, R and UA, three different types of treatments were performed: 1) cells were simultaneously exposed for 1 h to *t*-BHP and one test compound at different concentrations (ST); 2) pre-treatment (PT) with Q, R or UA at different concentrations for 24 h before medium change and exposure to *t*-BHP for 1 h; and, 3) pre-treatment with recovery period (PTR) – cells incubated as above and allowed a 2 h recovery period in fresh culture medium. A previous report (Aherne and O'Brien, 2000b) has shown that a 2 h recovery period is within the linear phase of single strand breaks repair.

Effects of the test compounds against *t*-BHP-induced DNA damage were evaluated by comet assay. In the PTR, DNA repair rate (RR) was calculated using the formula:

$RR = (D_0X\text{-}D_2X)/D_0X \times 100$

where D_0X represent DNA damage before recovery period in the condition X and D_2X represent DNA damage after 2 h recovery period in the condition X.

2.6. *Comet assay*

The alkaline version of the single cell gel electrophoresis assay was used to evaluate DNA damage as previously described by Lima et al. (2006) for HepG2 cells. For analysis of the comet images, the extent of DNA damage was estimated by fluorescence microscopy using the semiquantitative method of visual scoring (Duthie,

2003). Briefly, for each sample, 100 cells were analysed and classified visually into one of five classes according to the intensity of fluorescence (DNA) in the comet tail: class 0 (no damage) to class 4 (great damage, almost all DNA in tail). An overall score from 0 to 400 (arbitrary units) was attributed to each sample.

2.7. *Hsp 70 protein expression*

The effect of 24 h incubation with Q (50 µM), R (50 µM) or UA (25 µM) on Hsp70 expression was monitored by western blotting. A positive control was used where cells were heated at 42ºC for 1h, without test compounds, and allowed to recover for 16h at 37ºC. Protein concentration was measured with the Bradford Reagent and 20µg/well were separated on 8% SDS-PAGE and transferred to PVDF membranes. Membranes were blocked and incubated separately with each of the two monoclonal anti-HSP70s (1:4,000 dilution) for 1 h at room temperature and incubated with the secondary antibody diluted 1:30,000 for 1 h at room temperature. Band area intensity was quantified using the densitometry software SigmaScan 1.0 (Jandel Scientific, Scan Rafael, CA, USA). The results were expressed as percentage of control (cells without any test compound or heat treatment).

2.8. *Statistical analysis*

Results were expressed as mean ± SEM. Significant differences (P<0.05) within treatment groups were determined by one-way ANOVA followed by the Student-Newman-Keuls multiple comparison test. Between PT and PTR significant differences were determined by two-way ANOVA followed by the Student-Newman-Keuls multiple comparison test.

3. Results

3.1. Antiradical activity

The antiradical activity of the 3 tested compounds was evaluated by the ability to scavenge the free radical DPPH (Table 1). As expected, Q and R showed high antiradical activity, with Q showing the highest activity. Ursolic acid did not show the capacity to scavenge DPPH even at the high dose (200 µM).

3.2. Cytotoxic and antiproliferative effects

In order to establish dose and exposure times, preliminary evaluations of test compounds' toxicity were done using LDH leakage as indicator. Treatment for 24 h with the different concentrations of R and UA had no significant effect on extracellular LDH activity (Table 2). Q significantly decreased cell viability only at 100 μM (high concentration tested) when incubated for 24 h. When cells were incubated for 72 h, cell damage was apparent at concentrations higher than 25 μM for Q and higher than 50 μM for UA (Table 2). Rutin had no cytotoxic effects. In antigenotoxicity experiments compounds were used at non cytotoxic concentrations.

Effects on proliferation were also evaluated. Incubations with Q for 24 h and 72 h induced a concentration dependent inhibition of cell proliferation as determined by MTT assay (Fig. 2). At 24 h, inhibition was 30% and 45% at 25 and 50 μM of Q, respectively (Fig. 2A). Incubations for 72 h exhibited inhibitions of 40% and 86% at 12.5 and 25 μM of Q, respectively (Fig. 2B). Corroborating the LDH results, incubations with Q at 50 μM for 72 h induced cell death, as indicated by the negative value of cell proliferation seen by the MTT assay. Ursolic acid inhibited cell proliferation only at 50 μM when incubated for 72 h, showing 86% inhibition (Fig. 2B). Antiproliferative effects of Q and UA increased with increasing incubation time. R showed no effects on proliferation at any of the tested concentrations (Fig. 2).

3.3. Genotoxic effects of t-BHP

The concentration of 200 μM *t*-BHP was chosen to test (1h, 37ºC), the potential antigenotoxic effects of Q, R and UA. This dose and exposure time to the toxicant were chosen because it was not cytotoxic (data not shown) and produced intermediate levels of DNA damage (≈ 250 arbitrary units) detectable by the Comet assay (Fig. 3). HepG2 cells incubated for 1 h with or without 200 μM *t*-BHP were used as positive and negative controls, respectively.

3.4. Antigenotoxic effects of Q, R and UA

3.4.1 Simultaneous incubation with t-BHP

The antigenotoxic effects of Q, R or UA against *t*-BHP-induced DNA damage in simultaneous 1 h incubations were assessed by the comet assay, and Q showed a concentration-dependent chemoprotection against the oxidative-induced DNA damage (Fig. 4A). The extent of DNA damage was decreased by 20%, 41% and 57% by 12.5, 25 and 50 μM of Q, respectively. Contrarily, R and UA at the tested concentrations

were not effective in protecting DNA from *t*-BHP-induced damage under these incubation conditions (Figs. 4B and 4C). None of the test compounds at the higher tested concentration induced DNA damage when incubated without *t*-BHP although at higher concentration 100 μM Q has been reported to have genotoxic effects (Duthie et al., 1997). In the antigenotoxic experiments, the higher tested concentration of UA was 25 μM, since cell toxicity was observed for higher concentrations, as indicated by cell morphological observations (data not shown), in the experimental conditions used for the comet assay.

3.4.2. Pre-treatment with or without recovery period

The protection against *t*-BHP induced DNA damage conferred by a 24 h pre-treatment with Q, R or UA to HepG2 cells is shown in figure 5. In the experiments without recovery period (white bars), pre-treatment of HepG2 cells with Q resulted in lower levels of *t*-BHP-induced DNA damage (Fig. 5A). Quercetin at 25 and 50 μM significantly protected from DNA damage by 17% and 29%, respectively. No decrease in damage induced by *t*-BHP was observed in pre-treatment with R when compared to the positive control (Fig. 5C). In pre-incubations with UA, significant differences were found only for the highest tested concentration (25 μM) (Fig. 5B). The extent of DNA damage was, in this case, 20% smaller than control.

When the cells were subsequently allowed to recover for 2 h in fresh medium DNA damage decreased significantly in all cases ($P<0.001$) (Fig. 5 - grey bars). However, significant less DNA damage was observed in cells preincubated with Q (at all tested concentrations) and UA (at 12.5 μM) compared with the respective control (Figs. 5A and 5B) indicating a significant increase in DNA RR induced by these two compounds (Figs. 5A and 5B - insert). Rutin did not show any effect against *t*-BHP-induced DNA damages with or without recovery period (Fig. 5C).

3.5. HSP70 expression

To verify the potential involvement of Hsp70 on the antigenotoxic properties of compounds we evaluated the effects of Q, R or UA on Hsp70 expression in HepG2 cells. None of the test compounds at the tested concentrations showed a significant induction of Hsp70 expression in cell homogenates, using both the antibodies Sigma (Fig. 6) and Stressgen (inducible form; data not shown). However, heat shock treatment

261 of HepG2 cells (positive control) resulted in the expected induction of Hsp70
262 expression (Fig. 6).
263
264 **4. Discussion**

265 In this study, we proposed to evaluate the chemoprotective effects of natural
266 compounds common in fruits and vegetables, the structurally related flavonoids Q and
267 R as well as the triterpenoid UA, against DNA damage induced by *t*-BHP. Although all
268 have been reported to be hepatoprotective, there were major differences between the
269 three compounds with respect to antiradical activity: Q and R had high free radical
270 scavenging activity, whereas UA was virtually inactive as free radical scavenger at the
271 concentrations used (Table 1). In spite of this, Q and UA showed some protective
272 effects on cellular DNA but R did not, indicating that the hepatoprotective and
273 anticancer activities of these compounds may be the result of effects other than their
274 antioxidant activity alone. In this study, oxidative damage was inflicted to HepG2 cells
275 with *t*-BHP, which acts, by two distinct pathways. One involves cytochrome P450
276 and/or free iron ions leading to the formation of toxic alkoxyl and peroxyl radicals,
277 which can initiate lipid peroxidation, induce DNA damage, affect cell integrity and form
278 covalent bonds with cellular molecules, resulting in cell injury (Lin et al., 2000).
279 Alternatively, *t*-BHP can be metabolized by glutathione peroxidase (GPx) with
280 formation of oxidized glutathione (GSSG) from its reduced form (GSH). Depletion of
281 GSH and NADPH oxidation result and are associated with altered Ca^{2+} homeostasis,
282 which is considered a critical event in the *t*-BHP induced loss of cell viability (Lin et al.,
283 2000).

284 In order to distinguish possible differences in mechanisms of protection by the
285 natural test compounds, three types of incubations regimes were used: simultaneous
286 incubations; pretreatment and pretreatment with recovery period. In simultaneous
287 incubation only Q (and not R or UA) showed DNA protection whereas in pretreatment
288 experiments both Q and UA were active. Q and UA also showed induction of DNA
289 repair as evidenced by the recovery treatment. R was not active at any these levels.

290 In simultaneous incubation experiments natural compounds may protect against
291 oxidant-induced DNA damage directly either by free radical scavenging activity or by
292 decreasing free radical production through iron chelation (Anderson et al., 2000; Ross
293 and Kasum, 2002). The DNA single strand breaks caused by *t*-BHP have been

suggested to be the result of covalent binding of free radicals to DNA through iron dependent mechanisms (Latour et al., 1995; Sestili et al., 1998). In addition to their antiradical activity, both Q and R possess the structural features that enable them to chelate metal ions namely the ortho dihydroxy (catechol) phenolic structure (Rice-Evans et al., 1996). However, only Q was able to prevent *t*-BHP induced DNA damage in HepG2 cells in simultaneous incubations. This may be due to the lower hydrophobicity and bioavailability of R (Lima et al., 2006). Estimates of a compound's hydrophobicity can be made by using the KowWin (LogKow) software (assessable at http://www.syrres.com). The estimated Log P for Q is 1.48 and 7.92 for UA, whereas -2.02 is the value estimated for R. Although highly lipophilic, UA did not show DNA protection in simultaneous incubations, which may reflect its low free radical scavenging activity and low iron chelating capacity.

In pre-incubation experiments, in which the cells were exposed to the toxicant in fresh medium after a 24 h pre-incubation with the natural test compound, it is expect that the observed ability to prevent *t*-BHP-induced DNA damage is due to cellularly mediated effects such as increased enzymatic and non-enzymatic cellular antioxidants, inhibition of bioactivacting (phase I) enzymes and/or induction of detoxifying (phase II) enzymes. Both Q and UA showed chemoprotective activities against oxidative-induced DNA damage in this type of experiment. In others studies, pre-incubation with Q was also able of decrease DNA damage induced by hydrogen peroxide (Aherne and O'Brien, 1999; Duthie and Dobson, 1999). Several studies have show that Q as well other flavonoids can increase cell GSH content, the activity of antioxidant and phase 2 enzymes as well as inhibiting cythocromes P450's (Kang et al., 1999; Ferguson, 2001; Alía et al., 2005; Moon et al., 2006), this could explain our results. Also, UA has been shown to significantly increase levels of non-enzymatic (GSH) and enzymatic antioxidants as well as inhibiting cythocrome P450's (Liu, 1995; Martin-Aragón et al., 2001; Saravanan et al., 2005).

In pre-treatment with recovery period experiments, Q (12.5, 25, 50μM) and UA (12.5μM) showed significant induction of DNA repair contrarily to what happened in a study by Aherne and O'Brien (2000b). In that study using HepG2, Caco-2, and V79 cells, treated with H_2O_2, Q and R at 50 μM did not increase the rate of DNA repair. This discrepancy may reflect differences in the methodology such as oxidant used. Although nothing is known about UA's effects on DNA repair enzymes, polyphenols have been

suggested to influence DNA repair enzymes through modulation of gene expression (Ferguson, 2001; Ferguson et al., 2004).

In summary, the effects of Q seem to be due to effects at several levels that include radical scavenging, iron chelation, modulation of cell antioxidant responses as well as effects on DNA repair capacity. UA protects DNA indirectly through cellularly mediated effects, which include induction of DNA repair. In a recent study Doulias et al., (2007) showed that the stabilisation by Hsp70 of the lysossomal membrane may prevent the leakage of iron ions necessary for Fenton reaction-mediated DNA damage to occur. Some studies have also suggested the involvement of Hsp70 in the activation of DNA repair (Kenny et al., 2001; Mendez et al., 2003a) particularly of base excision repair (BER) enzymes, the type necessary to repair *t*-BHP induced damages. We therefore investigated whether UA effects could be due to Hsp70 induction and prevention of lysossomal iron leakage. Q on the other hand has been known to inhibit Hsp70 induction. We observed no changes on Hsp70 expression induced by Q, R or UA in HepG2. Although not induced by the test compounds Hsp70 was strongly induced by heat shock (used as a positive control). Our results indicate that UA is not an Hsp70 inducer and therefore, (as Q) seems not to be exerting its effects through this mechanism in HepG2 cells.

Damage to DNA may cause mutations that potentially lead to cancer (Ferguson, 2001; Ferguson et al., 2004). Therefore, protection against DNA damage and induction of DNA repair enzymes represent important mechanisms of anticarcinogenic activity of natural compounds. Other mechanisms of anticarcinogenicity include inhibition of damaged cell proliferation by cell cycle arrest and/or induction of apoptosis (Birt et al., 2001). In our study, both Q and UA but not R showed antiproliferative effects in HepG2 cells. These are in agreement with previous reports that showed a generalized growth inhibitory effect of Q and UA on several cancer cell lines (Novotny et al., 2001; Alía et al., 2005; Nichenametla et al., 2006; Tian et al., 2006). For longer exposure periods to higher concentrations of Q and UA, the antiproliferative effects were also accompanied by cell toxicity. In agreement with other results using different cell lines (Kuo, 1996; Kim et al., 2005), R did not show antiproliferative effects in HepG2, contrarily to what happened in study by Alía et al. (2005) using HepG2 cells, in which different cell culture conditions were used. Nevertheless, in an *in vivo* situation, dietary R can still play a role in the chemoprevention, since it is known that it can be deglycosylated to yield quercetin in the intestine by colon microflora (Kuo, 1996).

In conclusion, in the present study the anticarcinogenic potential of Q and UA were shown through their antigenotoxic and antiproliferative activities on HepG2 cells. The antigenotoxic effects of Q against *t*-BHP seems to be due both to direct effects and cellularly mediated indirect effects, whereas effects of UA are cell mediated. Induction of DNA repair by Q and UA may also contribute for the antigenotoxic effects of these compounds, although more work is necessary to further characterise the effects of these compounds at this level. Their prevention of oxidative DNA damage may help explain the cancer chemopreventive effects associated to a high fruit and vegetable diet.

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Tables

Table 1 – Antiradical activity of Q, R and UA as determined by the DPPH assay.

Compound	IC_{50} (μM)
Quercetin	11.34 ± 0.04
Rutin	18.27 ± 0.62
Ursolic Acid *	-
Trolox	18.04 ± 0.01

* Maximal concentration tested - 200μM

Table 2 – Effects of Q, R and UA on cellular viability (as % of control) of HepG2 cells as measured by LDH leakage. Incubations were performed for 24h or 72h with different natural compounds concentrations. Mean ± SEM (n=4). * $P<0.05$ and *** $P<0.001$ when compared to the respective control.

Concentration (μM)	Cellular Viability (% of control)					
	24h			72h		
	Q	R	UA	Q	R	UA
0	100.0±1.1	100.0±0.3	100.0±1.0	100.0±1.0	100.0±0.7	100.0±0.7
12.5	103.1±1.3	99.9±2.6	100.5±1.1	100.0±0.6	101.4±0.8	100.8±0.2
25	101.1±0.8	103.0±0.4	102.6±1.1	90.5±1.2***	102.1±0.6	100.0±0.4
50	99.4±0.6	101.0±0.7	101.5±1.4	72.4±1.2***	102.2±0.5	93.0±1.2***
100	95.1±0.5*	100.7±0.4	95.7±1.6	71.9±0.4***	102.4±0.8	65.4±1.3***

Figure legends

Fig. 1 – Chemical structures of Q and R (A) as well as UA (B).

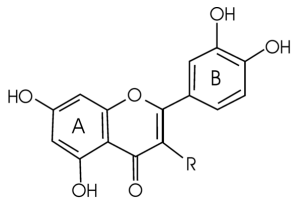
Fig. 2 – Effect of incubation for 24h of Q, R and UA (with a subsequent 48h period with fresh culture medium without natural compounds) (A) and for 72h (B) on HepG2 proliferation measured by the MTT assay. Mean ± SEM of at least three independent experiments (5 replicates each). * $P<0.05$ and *** $P<0.001$ when compared to the respective control.

Fig. 3 – Effect of *t*-BHP on DNA stability of HepG2 cells. DNA damage was assessed by the comet assay. Mean ± SEM (n=4). *** $P<0.001$ when compared to control.

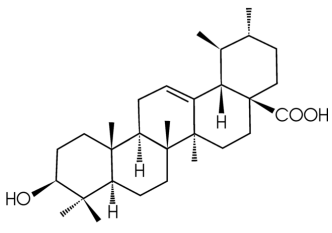
Fig. 4 – Effect of Q (A), UA (B) and R (C) against *t*-BHP (200µM, 1h)-induced DNA damage in HepG2 cells, estimated by the comet assay. Mean ± SEM (n=4). ### $P<0.001$ when compared to negative control; *** $P<0.001$ when compared to positive control.

Fig. 5 – Effect of 24h pre-treatment of Q (A) UA (B) and R (C) on *t*-BHP (200µM, 1hr)-induced DNA damage in HepG2 cells with (grey bars) or without (white bars) 2h of recovery period. DNA damage was assessed by the comet assay. DNA repair rate of Q, UA and R (respective insert) was calculated with the formula describe in material and methods section. Mean ± SEM (n=4). ### $P<0.001$ when compared to respective negative control; * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ when compared to respective positive control.

Fig. 6 – The effect of 24 h incubation with Q (50 µM), R (50 µM) or UA (25 µM) on the expression of Hsp70, as estimated by Western blotting. In the positive control, HepG2 cells were heated at 42ºC for 1h and allowed to recover for 16h at 37ºC. Mean ± SEM (n=3). *** $P<0.001$ when compared to control.



Compound	R
Quercetin	OH
Rutin	O-rutinose



Ursolic acid